Identification of Imidacloprid Metabolites in Onions Using High Resolution Accurate Mass Spectrometry (LC/Q-TOF MS) and Accurate Mass Tools

Application Note

Food

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Abstract

The use of the Agilent 6540 Q-TOF LC/MS system and Agilent MassHunter software tools have enabled the identification of six new metabolites of imidacloprid by MS/MS analysis. These metabolites and their structures were deduced from MS/MS studies with accurate mass, making it possible to better understand imidacloprid metabolism in onion as well as new metabolite targets for toxicity studies.

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Introduction

This application note describes tools used in a more complete technical report from this work [1]. Imidacloprid is a neonicotinoid pesticide that was introduced to the market in the late 1990s for the control of homopteran pests, such as aphids, planthoppers, and whiteflies, as well as certain beetles. It has become one of the most widely used insecticides, particularly for agriculture. Imidacloprid acts as an agonist of the nicotinoid acetylcholine receptor, which is highly specific to insects [2,3].

Imidacloprid is one of the most toxic insecticides to bees. Research suggests that widespread agricultural use of imidacloprid and other pesticides may be contributing to the decline of honey bee colonies in Europe and North America observed since 2006. Thus, imidacloprid is an important insecticide to investigate in plant fate and metabolism studies [4].

Although imidacloprid metabolism has been studied in a number of crops, it has not been studied in detail in onions. This application note describes the methodology and tools used for the determination of imidacloprid metabolites in onions using ultra-high performance liquid chromatography (UHPLC)/quadrupole time-of-flight mass spectrometry (Q-TOF MS) in MS, All Ions MS/MS, and targeted MS/MS modes. Several accurate mass tools were used to identify the metabolites, including six that had not been previously reported. Using this methodology, the distribution of imidacloprid metabolites between the onion plants, the soil, and leachate was determined.

Experimental

Reagents and standards

An individual pesticide stock solution (approximately 1,000 µg/mL) was prepared in methanol and stored at –18 °C. From this solution, working standard solutions were prepared by dilution with acetonitrile and water, as described previously [1]. The guanidine metabolite was synthesized by hydrolysis of imidacloprid in 1.0 N HCl for 1 hour at 45 °C, also as described previously [1].

Instruments

This study was conducted using an Agilent 1290 Infinity LC System coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS system equipped with electrospray Jet Stream Technology. The run conditions are shown in Table 1.

Table 1. LC and Q-TOF MS Run Conditions

LC run conditions	
Column	Analytical column: Agilent ZORBAX Eclipse XDB C8 Reversed-Phase, 4.6 × 150 mm, 3.5 µm (p/n 963967-906)
Column temperature	25 °C
Injection volume	20 µL
Mobile phase	A = 0.1% formic acid in water v/v B = Acetonitrile
Linear gradient	10% B for 5 minutes, then 10% B to 100% B for 25 minutes at constant flow, then hold at 100% B for 10 minutes
Flow rate	0.6 mL/min
Q-TOF MS conditions	
Ion mode	ESI, positive ion mode
Nebulizer pressure	45 psig
Drying gas flow rate	10 L/min
Drying gas temperature	250 °C
Sheath gas flow rate	11 L/min
Sheath gas temperature	350 °C
Nozzle voltage	0 V in positive ion mode
Fragmentor voltage	190 V
Capillary voltage	3,500 V
Mass range	50–1,000 *m/z*
Detector rate	2 GHz
Resolving power	35,000 ± 500
Accuracy	2 ppm

Pesticide application

Onions (*Allium cepa L.*) were grown from certified seed in a greenhouse environment as previously described [1]. Imidacloprid was applied to each plant at 2.5 µg/mL in 200 mL of water.

Sample preparation

Water samples (leachate) were collected once a week for three weeks. Soil and plant samples were collected in triplicate at 28, 38, and 53 days, for a total of 36 samples each of soil and plants. Water samples were filtered, if necessary, and spiked with deuterated imidacloprid. Soil and plant extractions were performed as shown in Figure 1.

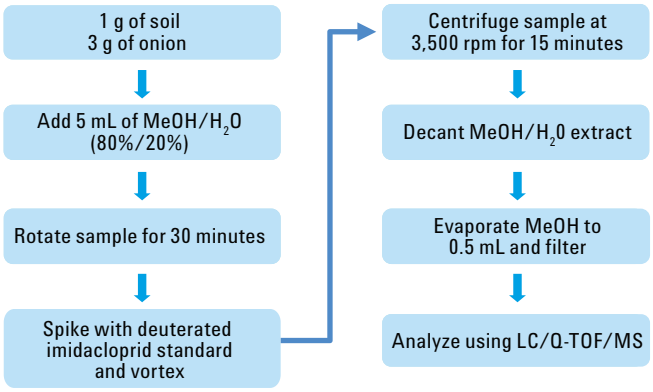


Figure 1. Flow chart of procedure used to extract soil and plant samples.

Data analysis

Several Agilent software tools were used to aid in identifying imidacloprid metabolites, including Molecular Feature Extractor, Molecular Formula Generator, Mass Profiler, and accurate mass databases. Author-constructed adaptations of the software [5] were also used, including the Chlorine Filter, as the parent compound was chlorinated [6].

Results and Discussion

Database search for known metabolites

A typical total ion chromatogram (TIC) of the onion extract is very complex, making it very difficult to identify any imidacloprid metabolites (Figure 2A). However, a database search for imidacloprid and its 12 known metabolites reveals the presence of at least two peaks that may correspond to the parent compound (*m/z* 256.0594 measured) and the guanidine metabolite (*m/z* 211.0743 measured) as shown in the extracted ion chromatogram (EIC) of the onion extract (Figure 2B). MS/MS analysis of the *m/z* 256 peak identified it as imidacloprid [1].

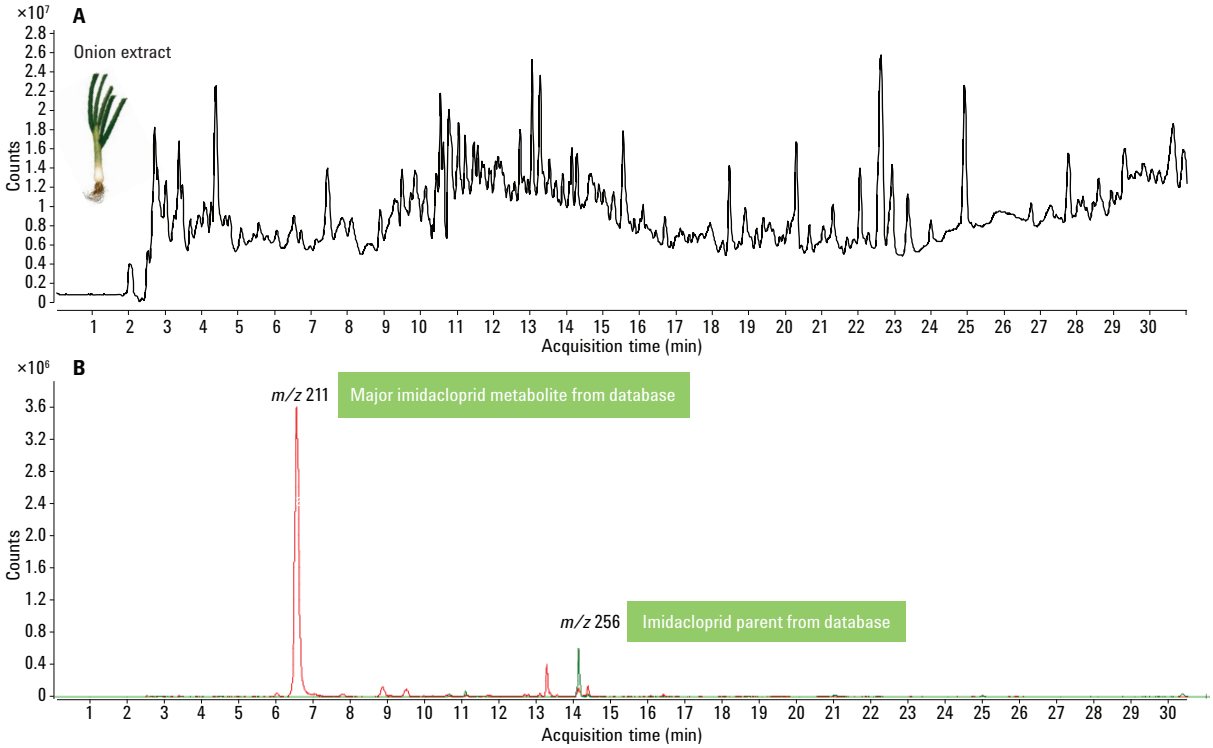


Figure 2. Typical TIC for onion extract of an onion plant harvested at 28 days treated with imidacloprid (A) and EIC for several known metabolites of imidacloprid showing major peaks for the parent compound and the m/z 211 guanidine metabolite (B).

The *m/z* 211.0743 peak was within 1 ppm of the calculated exact mass of the guanidine metabolite, and MS/MS analysis of this peak provided its identity [1]. Comparison to the standards of these compounds confirmed their identities.

The exact mass database search detected two more of the 12 known metabolites, 4-hydroxyimidacloprid and the urea analogue of imidacloprid. None of the other nine known metabolites were present in the onion extracts based on accurate mass and extracted ion chromatograms within a 5-ppm accurate mass window [1].

Diagnostic ions and All Ions MS/MS

Diagnostic fragment ions refer to moieties of the imidacloprid structure that are consistent in compounds with related structures and typically present in their metabolites. These may be produced with fragmentation with the All Ions MS/MS approach, allowing diagnostic ion search (DIS) across the chromatogram. This is done by either alternating the fragmentor (TOF or Q-TOF) from a low to high voltage, or alternating the collision energy from 0 to a moderate collision energy (Q-TOF only with quadrupole in Rf only mode) to generate the diagnostic ion of interest, which can then be used to identify potentially new metabolites.

An ion at *m/z* 126 was generated from the guanidine metabolite of imidacloprid upon MS/MS, and it is part of the imidacloprid parent compound structure (Figure 3). Performing DIS on the onion extract from plants treated with imidacloprid using the *m/z* 126 ion identifies a few peaks that may be putative metabolites (Figure 3). One of these was the guanidine analogue, (retention time, or RT, 6.6 minutes) a known metabolite. A shoulder peak at *m/z* 209.0589 (RT 6.4 minutes) was identified as the olefin of the guanidine analogue, a newly observed metabolite [1], by subsequent MS/MS. The third peak at *m/z* 226.0845 (RT 6.0 minutes) was identified by MS/MS as the reduced amine analogue of imidacloprid. This metabolite also had not been reported previously [1]. Onion plants not treated with imidacloprid did not exhibit any peaks at *m/z* 126 upon DIS.

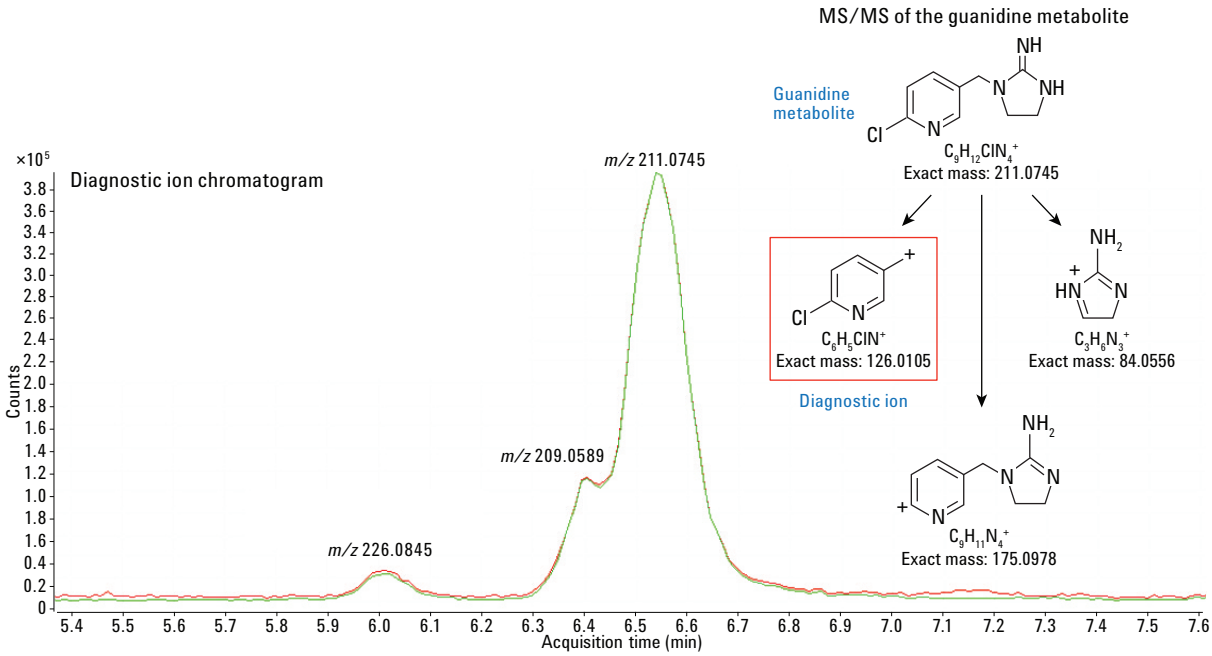


Figure 3. Diagnostic ion of All Ions MS/MS analysis of an onion extract treated with imidacloprid, using the m/z 126 ion that is an MS/MS product of the guanidine metabolite and part of the imidacloprid structure. The plant sample was collected 28 days after treatment with the pesticide. The collision voltage was set at 0 and 30 eV.

Metabolic discovery using the Chlorine Filter

The Chlorine Filter [6] in MassHunter Software is another tool used to identify new metabolites. First, the Molecular Feature Extractor was used to find compounds in the chromatogram above an ion intensity of 10,000 counts. Then, molecular formulas containing only one chlorine atom for the extracted compounds were generated, since imidacloprid contains only one chlorine atom. When the chlorine filter was run on the onion extracts, several new putative metabolites were found that were not found by either the database search or the diagnostic ion search (Figure 4).

One of the compounds, found only by the chlorine filter, had a mass of *m/z* 354.0955 and a retention time of 10.96 minutes, 98 mass units larger than imidacloprid. The formula contains five more carbons and two more oxygen atoms than the parent compound, while the chlorine and nitrogen content are the same as the parent, suggesting conjugation with another group.

Conjugates with glutathione, a tripeptide consisting of cysteine, glycine, and glutamic acid have been reported with pesticides, but not with imidacloprid. While glutathione would add too much mass to account for the new metabolite, glutamic acid alone does contain the additional five carbons. However, glutamic acid would add 147 mass units, not 98.

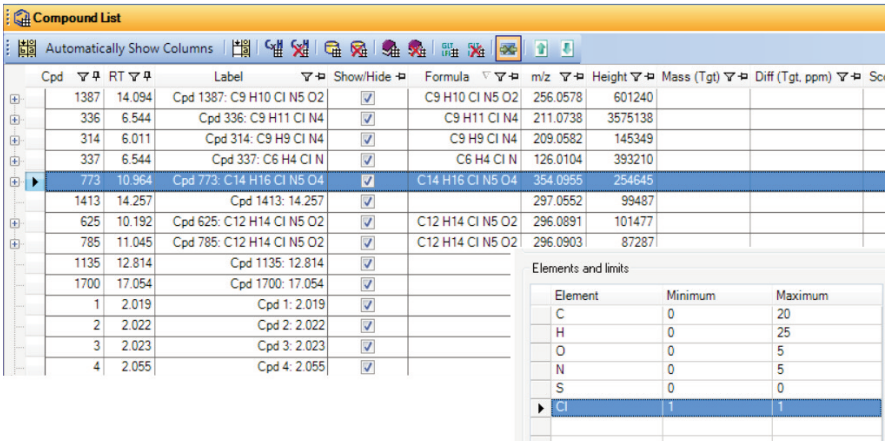


Figure 4. Chlorine filter generating molecular formulae constrained to contain only one chlorine atom. Several metabolites were determined with this tool, including a new metabolite (highlighted) with an accurate mass measured at m/z 354.0955.

Analysis of this compound by MS/MS revealed neutral losses of two 46 amu moieties (Figure 5). The measured mass of each of these losses can only be $COOH_2$ (formic acid). The reduction of the nitro group with glutamic acid fits this data. Another major fragment is the *m/z* 209.0550 (measured accurate mass) ion, which can also form by the loss of glutamic acid (Figure 5). This data identifies the structure of the new metabolite as the glutamic acid conjugate of imidacloprid.

Another of the compounds found by the chlorine filter, with *m/z* 240.0996, gave the formula $C_{10}H_{14}ClN_5$, which is consistent with the addition of a methyl group to the metabolite at *m/z* 226 (amine analog of imidacloprid), and was identified as methyl imidacloprid by MS/MS [1]. Methylation is commonly seen among onion plants for DNA, but not for pesticides. This putative structure represents another new and possibly toxic metabolite of imidacloprid, based on previous reasoning.

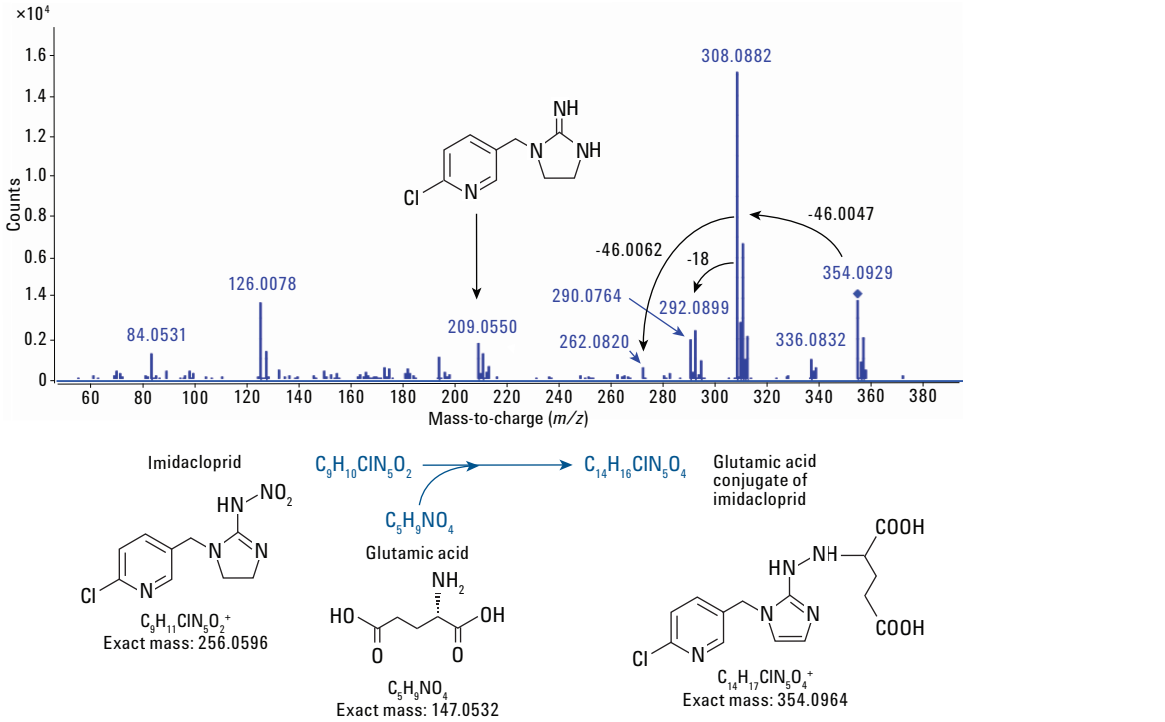


Figure 5. Using MS/MS transitions and knowledge of plant metabolism to determine the structure of the m/z 354.0955 metabolite. The neutral loss of two 46 amu moieties can only be explained as loss of two $COOH_2$ (formic acid) groups. Reduction of the nitro group on imidacloprid by glutamic acid accounts for the two carboxyl groups on the metabolite and the gain of five carbons and six hydrogens by imidacloprid, to give the correct mass of the metabolite.

Distinguishing differences with mass profiler

Agilent Mass Profiler software was used to compare all of the compounds found by the molecular feature extractor in the pesticide-treated plants with those found in the control, nontreated plants (Figure 6). While the profiles of the two plant extracts are too complex to pull out putative metabolites, the results suggest that treatment of the plant with pesticide causes a large number of changes in the chemical composition of the plant, in addition to metabolism of the pesticide itself. This is an apparent phenomenon that could warrant additional study of the impact of pesticides, both positive and negative, on the nutritional content of plants.

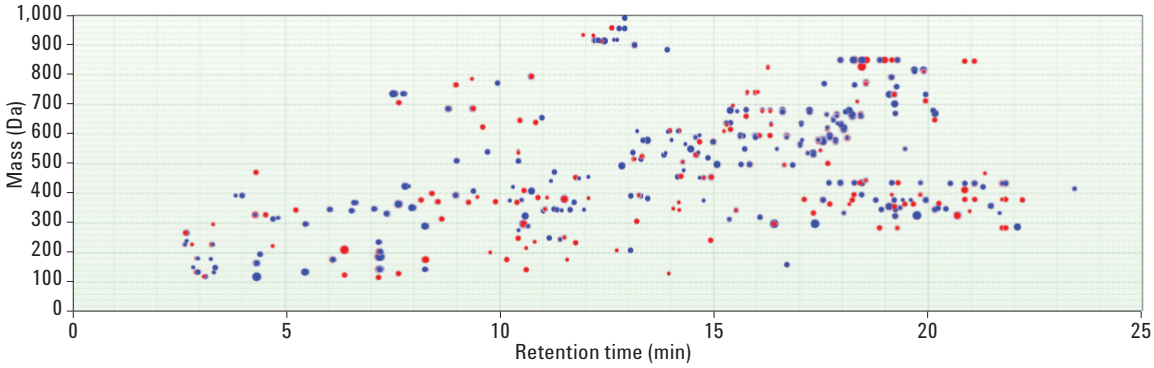
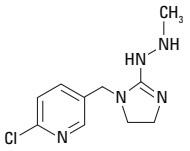
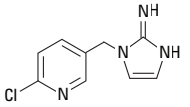
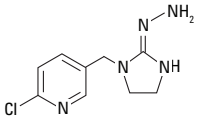
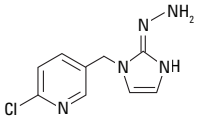
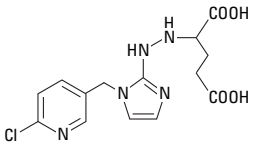
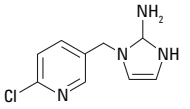


Figure 6. Comparison of the mass versus retention time for the average of three blank, untreated onion plant extracts, versus the average of three samples treated with imidacloprid and harvested at 28 days (Blue dots are common features to both sets of samples and red dots are unique to treated samples).

Distribution of Imidacloprid and it metabolites

Six new metabolites of imidacloprid were identified in this study (Table 2). Analysis of the plant and soil extracts for imidacloprid and these six metabolites revealed that the plant contains primarily the guanidine metabolite, and the soil contains primarily the unmetabolized pesticide. The other five new metabolites are all present in trace amounts, relative to the guanidine metabolite and the parent pesticide (Figure 7).

Table 2. Six Newly Identified Imidacloprid Metabolites by MS/MS Analysis

Metabolites	Elemental composition	Retention time (min)	Exact mass $[M+H]^+$	Chemical structure
Methylated imidacloprid	$C_{10}H_{14}ClN_5$	8.3	240.1010	
Olefin of guanidine analogue	$C_9H_9ClN_4$	6.4	209.0589	
Imidacloprid-amine analogue	$C_9H_{12}ClN_5$	6.0	226.0854	
Olefin-imidacloprid-amine analogue	$C_9H_{10}ClN_5$	4.8	224.0697	
Glutamic acid conjugate of imidacloprid olefin guanidine	$C_{14}H_{16}ClN_5O_4$	10.7	354.0964	
Isomer of guanidine imidacloprid	$C_9H_{11}ClN_4$	7.1	211.0745	

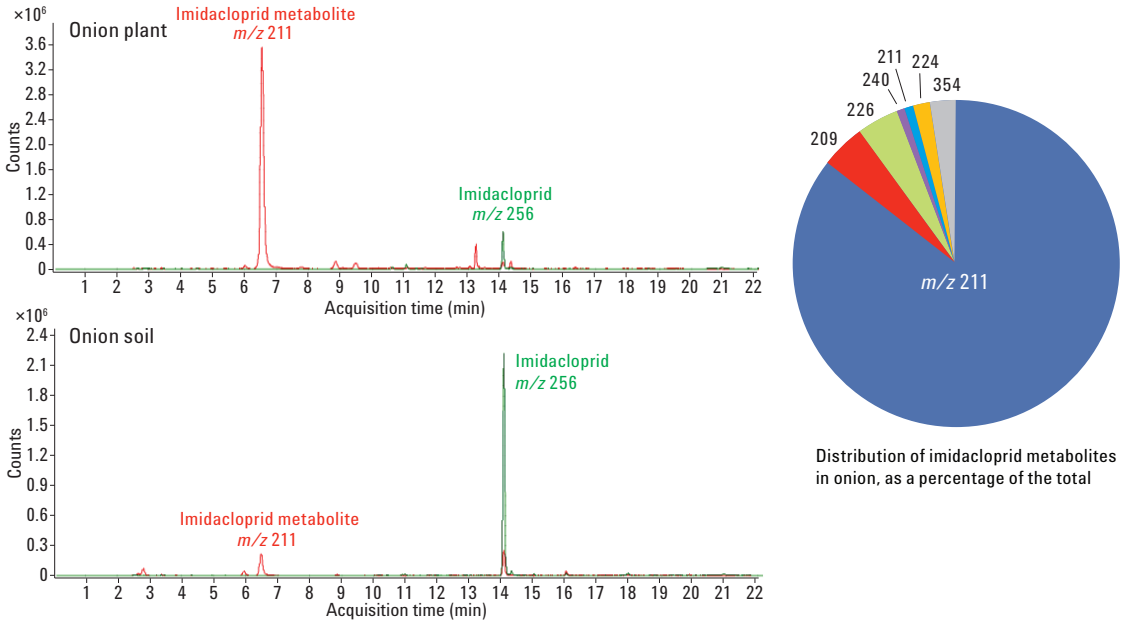


Figure 7. Distribution of the pesticide and its main metabolite, the guanidine analogue (m/z 211) in both soil and plant extracts. The pie chart shows the distribution in the onion plant of the five newly identified metabolites plus the guanidine analogue (m/z 211), as a percentage of the total of the seven metabolites, including the two isomers of the guanidine analogue.

Conclusions

The Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS system and software provide powerful tools for identifying and characterizing metabolites of imidacloprid, in addition to enabling detailed studies of the fate of the pesticide in the plant and the environment. Using such tools as accurate mass database searches, diagnostic ions with All Ions MS/MS, the chlorine filter, and Mass Profiler, new metabolites can be elucidated with little or no prior knowledge of their structure. These tools have general applicability to any study that requires identification of a large number of previously unknown derivatives of a parent compound that occur at moderate intensity in complex samples.

References

1. E.M. Thurman, *et al.* "Identification of imidacloprid metabolites in onion (*Allium cepa L.*) using high-resolution mass spectrometry and accurate mass tools" *Rapid Commun Mass Spectrom.* **27**(17):1891-903 (2013).

2. S. Kagabu "Discovery of imidacloprid and further developments from strategic molecular designs" *J. Agric. Food Chem.* **59**, 2887- 2896 (2011).

3. M. Tomizawa, J.E. Casida "Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors" *Annu. Rev. Entomol.* **48**, 339-364 (2003).

4. T. Iwasa, *et al.* "Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*" *Crop Protection* **23**, 371-378 (2004).

5. E.M. Thurman and I. Ferrer "The isotopic mass defect: A tool for limiting molecular formula by accurate mass" *Anal. Bioanal. Chem.* **397**, 2807-2816 (2010).

6. I. Ferrer and E.M. Thurman "Identification of a new anti-depressant and its glucuronide metabolite in water samples using liquid chromatography/quadrupole time-of-flight mass spectrometry" *Anal. Chem.* **82**, 8161-8168 (2010).

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